PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN THE APPLICATION OF:

SAVERIO C. FALCO
SHARON J. KEELER
JANET A. RICE

CASE NO.: BB-1037-D

APPLN. NO.: 08/823,771

GROUP ART UNIT: 1638

FILED: MARCH 24, 1997

EXAMINER: E. MCELWAIN

FOR: CHIMERIC GENES AND METHODS FOR INCREASING THE LYSINE AND THREONINE CONTENT OF THE SEEDS OF PLANTS

Date: FEBRUARY 16, 2001

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

Declaration of Dr. Carl Falco Pursuant to 37 CFR §1.132

I, Saverio Carl Falco, am a citizen of the United States of America, residing at 1902 Millers Road, Arden, Delaware 19810, United States of America, and I declare as follows:

1. I am one of the above-identified inventors named in this application. I am a graduate of Rutgers University of New Brunswick, New Jersey with a B.A. degree granted in 1971 with high honors and distinction in physics. I received a Ph.D. in 1977 from the University of Chicago in biochemistry and molecular biology. From 1977 to 1981 I was a National Institutes of Health postdoctoral fellow at the Massachusetts Institute of Technology. I have been employed by E. I. du Pont de Nemours and Company since 1981 directing and conducting research in plant genetic engineering.

2. I have reviewed the Office Action dated November 22, 2000. I am aware that this declaration is being submitted to address the concerns set forth on page 3 of the Office Action that the "Declaration of Falco teaches use of a bifunctional LKR/SDH gene to identify mutants produced by transposon mutagenesis. This plant does not contain a foreign LKR gene. In addition, the Declaration of Falco teaches of a combination DHDPS gene without an AK gene. Thus, the Declaration of Falco

does not teach a plant with a foreign LKR gene and a foreign DHDPS gene . . . it remains unpredictable what the results would be of introducing just the LKR gene and the DHDPS gene into a plant."

3. It was stated in paragraph 10 of my declaration previously submitted on August 24, 2000 that a co-transformation experiment in which a chimeric gene designed for co-suppression of LKR was combined with a chimeric gene for expression of lysine insensitive DHDPS was in progress. That experiment was expected to yield transformants that produced seeds with higher free lysine levels than transformants from a parallel experiment using the DHDPS gene alone. The results of those experiments have now been obtained and they do confirm the prediction that transformants comprising the chimeric gene designed for co-suppression of LKR and the chimeric gene for expression of lysine insensitive DHDPS produced seeds with higher free lysine levels than transformants from a parallel experiment using the DHDPS gene alone. These results are depicted in Figure 2 and Table 1.

4. The chimeric genes used for the experiments were:

i) corn globulin1 promoter/corn chloroplast transit sequence/ Corynebacterium dapA gene/corn globulin1 3'UTR; and

ii) corn 27kd zein promoter/fragment of corn LKR-SDH cDNA/corn 10kd zein 3' UTR

Seeds from many transformation events from each experiment were analyzed for free lysine content. It is clear from the data presented in Figure 2 that the best seeds obtained from the co-transformation experiment had considerably higher free lysine levels than the best seeds obtained from the transformation experiment where only the DHDPS gene was used. The average free lysine level from the 30 highest lysine seeds, or from the 70 highest lysine seeds, was about 2-fold higher for the co-transformation experiments compared the DHDPS only experiment.

5. It also was stated in paragraph 10 of my previous declaration submitted on August 24, 2000 that an LKR co-suppression transformant which showed an increased seed free lysine phenotype for several generations, and behaved genetically as a single locus transgene insertion, was crossed to a transgenic line that accumulates excess free lysine due to expression of lysine insensitive DHDPS and AK. Results from that experiment, which were not available at the time of the previous declaration, have confirmed the expectations expressed there, namely that seeds carrying both transgene loci will have higher free lysine levels than either parent. The data are presented in Figure 1.

6. In this experiment described in paragraph 5 above, transgenic lines homozygous for an insertion of DHDPS and AK genes, or homozygous for the co-suppressing LKR/SDH gene, were each crossed to a wild type corn line or to each

other. The F1 progeny seed from these crosses are hemizygous for the DHDPS and AK transgenic insertion, the co-suppressing LKR/SDH transgenic insertion, or both. Each cross was repeated at least 5 times, and seeds from the resulting corn ears were harvested and analyzed for free lysine levels. The results depicted in Figure 1 are averages derived from these repetitions. These results show the dramatic increase in free lysine resulting from the combination of increasing the synthesis of lysine via expression of the DHDPS gene and blocking the major pathway for lysine catabolism by co-suppressing the LKR/SDH gene.

7. Parenthetically, it is noted that a concern was raised in the Office Action dated November 22, 2000 that results from combining the DHDPS and AK transgenic insertions with a co-suppressing LKR/SDH transgenic insertion would not be predictive of combining a DHDPS only transgenic insertion with a co-suppressing LKR/SDH transgenic insertion. It is noted that there is evidence in the subject application that AK plays a secondary role to DHDPS for increasing the synthesis of lysine.

For example, it was demonstrated for (i) rapeseed transformants on page 31 at lines 18 – 24 of the specification that :

"Transformants expressing DHDPS protein showed a greater than 100-fold increase in free lysine level in their seeds. There was a good correlation between transformants expressing higher levels of DHDPS protein and those having higher levels of free lysine. One transformant that expressed AKIII-M4 in the absence of *Corynebacteria* DHDPS showed a 5-fold increase in the level of free threonine in the seeds. Concomitant expression of both enzymes resulted in accumulation of high levels of free lysine, but not threonine."

And for (ii) corn transformants (page 33 at lines 15 – 24:

"Free lysine levels in the seeds is increased from about 1.4% of free amino acids in control seeds to 15-27% in seeds of transformants expressing *Corynebacterium* DHDPS alone from the globulin 1 promoter. The increased free lysine was localized to the embryo in seeds expressing *Corynebacterium* DHDPS from the globulin 1 promoter.

The large increases in free lysine result in significant increases in the total seed lysine content. Total lysine levels can be increased at least 130% in seeds expressing *Corynebacterium* DHDPS from the globulin 1 promoter. . . . Greater increases in free lysine levels can be achieved by expressing *E. coli* AKIII-M4 protein from the globulin 1 promoter in concert with *Corynebacterium* DHDPS."

8. Thus, the gene encoding lysine insensitive AK can enhance the effect of the DHDPS gene on lysine synthesis by increasing overall flux through the biosynthetic pathway. However, AK does not increase lysine when expressed without DHDPS. It is the DHDPS gene that is necessary for increasing the synthesis of lysine. The presence of the AK gene along with the DHDPS gene in the cross described above is inconsequential with respect to proof of the concept that the **combination** of increasing lysine synthesis (which can be achieved using the DHDPS gene alone or in combination with the AK gene) and blocking lysine catabolism (which can be achieved by blocking expression of the LKR/SDH gene via co-suppression) works better than either alone.

9. The genetic cross experiment and the co-transformation experiment described above, taken together with the detailed description of the invention provided in the patent application and the previous declaration, clearly demonstrate that an increased lysine content is achieved when a lysine insensitive DHDPS gene (with or without a lysine insensitive AK gene) is combined with a co-suppressing LKR gene.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Saverio Carl Falco

Feb 16, 2001

Date

Figure 1: Compare DHDPS + AK, csLKR, DHDPS + AK + csLKR

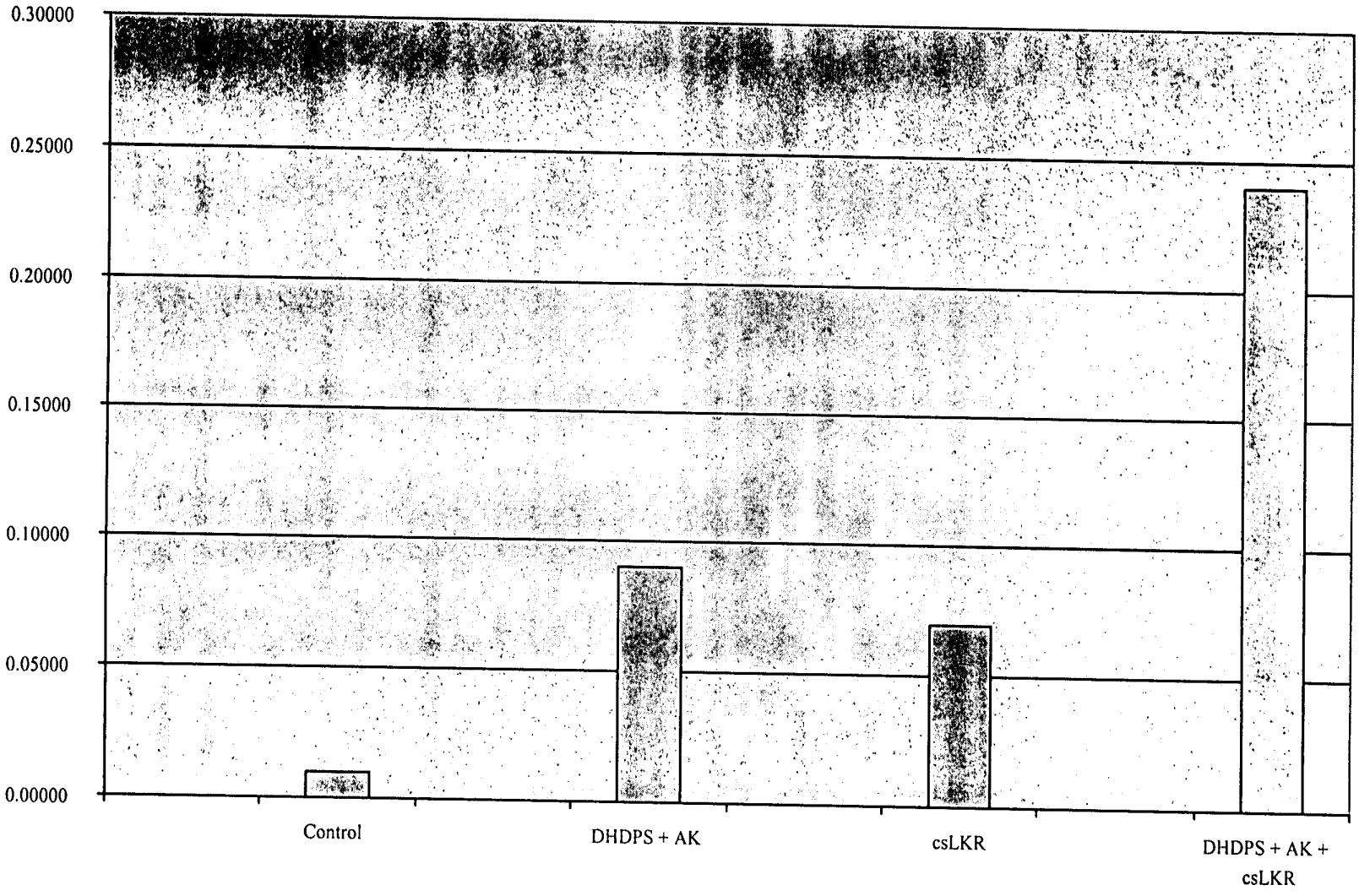

Figure 2: Comparison of seeds from transformation of DHDPS alone vs DHDPS + csLKR

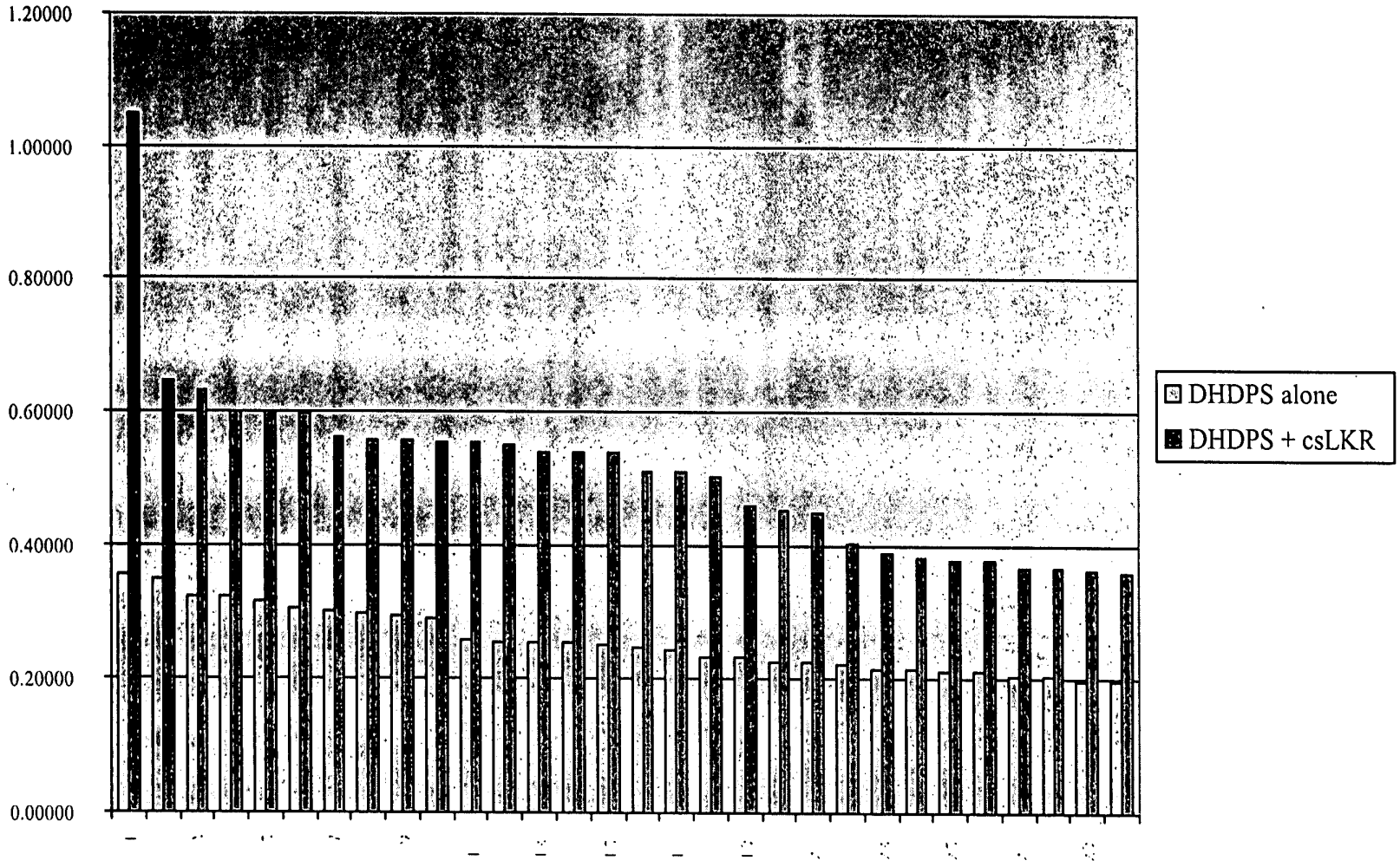

Table 1

	DHDPS alone wt % Free Lys	DHDPS + csLKR wt % Free Lys	wild type corn wt % Free Lys
Avg of best 30 seeds	0.26	0.51	0.01
Avg of best 70 seeds	0.20	0.39	0.01

Ag Case

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Date: AUGUST 24, 2000

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I, Saverio Carl Falco, am a citizen of the United States of America, residing at 1902 Miller Road, Arden, Delaware 19810, United States of America, and I declare as follows:

1. I am one of the above-identified inventors named in this application. I am a graduate of Rutgers University of New Brunswick, New Jersey with a B.A. degree granted in 1971 with high honors and distinction in physics. I received a Ph.D. in 1977 from the University of Chicago in biochemistry and molecular biology. From 1977 to 1981 I was a National Institutes of Health postdoctoral fellow at the Massachusetts Institute of Technology. I have been employed by E. I. du Pont de Nemours and Company since 1981 directing and conducting research in plant genetic engineering.

2. I have reviewed the Office Action dated April 25, 2000. I am aware that this declaration is being submitted to address the concerns set forth on page 4 and 5 of the Office Action that "the specification does not disclose any plants that comprise the claimed two gene fragments that result in the claimed increase in lysine relative to a

plant that does not comprise said two gene fragments. In addition, the specification fails to provide guidance with regard to the choice of subfragments that will result in the antisense inhibition or cosuppression of LKR."

3. At the outset, it is noted that many components of the process of plant genetic engineering, e.g. construction of chimeric genes for expression in plant cells, or for blocking expression of endogenous genes, transformation of plants, have become routine for those skilled in the art. Notwithstanding this, what follows is intended to show that one of ordinary skill in the art could follow the teachings of the instant application to practice the claimed invention without engaging in undue experimentation.

4. First, the rationale for combining the nucleic acid fragments of the invention are clearly disclosed in the specification. It was shown, for the first time, that accumulation of excess free lysine in plant seeds, accomplished via expression of lysine insensitive DHDPS, is accompanied by breakdown of free lysine and accumulation of intermediates in the breakdown pathway such as saccharopine. Thus, there was a clear incentive to reduce the loss of excess lysine due to catabolism.

5. Second, methods were provided to prevent lysine catabolism through reduction in the activity of the enzyme lysine ketoglutarate reductase (LKR), which catalyzes the first step in lysine breakdown. This can be accomplished by introducing a mutation in the plant gene that encodes LKR that reduces or eliminates enzyme function. Such mutations can be identified by screening mutants for lysine over-producer lines that do not accumulate the lysine breakdown products, saccharopine and α-amino adipic acid. Alternatively, the first nucleic acid fragments containing plant LKR cDNAs were disclosed. The nucleotide sequences of these fragments make it straightforward to isolate LKR nucleic acid fragments from any plant desired (see point 6 below). Chimeric genes for expression of antisense LKR RNA or for cosuppression of LKR in the seeds of plants can then be created. The chimeric LKR gene can be linked to chimeric genes encoding lysine insensitive AK and DHDPS and all introduced into plants via transformation simultaneously, or the chimeric LKR gene or mutant LKR gene can be brought together with chimeric genes encoding lysine insensitive AK and DHDPS by crossing plants to create hybrids carrying two or more of the genes (see below).

6. Third, examples of all of the nucleic acid fragments of the invention were provided in the specification of the subject case. In the case of the bifunctional protein lysine ketoglutarate reductase (LKR)/saccharopine dehydrogenase (SDH), two plant nucleic acid fragments (SEQ ID NOS:102 and 103) containing cDNA derived

from the plant *Arabidopsis thaliana* were provided in the present patent application. In the application it was stated that full length cDNAs encoding plant LKR plus saccharopine dehydrogenase (SDH) or genomic DNAs containing the entire LKR/SDH gene can be readily identified by hybridization to labelled cDNA fragments of SEQ ID NO:102: or SEQ ID NO:103: and thus isolated. This was, in fact, accomplished and is described in Epelbaum, S., McDevitt, R. and Falco, S. C., (1997) "Lysine-ketoglutarate reductase and saccharopine dehydrogenase from *Arabidopsis thaliana*: nucleotide sequence and characterization", Plant Mol. Biol. 35, 735.

The availability of the Arabidopsis LKR/SDH gene made it straightforward for us, as it would be for anyone skilled in the art, to isolate other plant LKR/SDH genes. Degenerate oligonucleotides were designed based upon highly conserved regions of the deduced amino acid sequence of plant and fungal proteins and used to amplify soybean and corn LKR/SDH cDNA fragments. Near full-length cDNAs for soybean and corn LKR/SDH were then isolated using 5' RACE and hybridization to cDNA libraries. LKR/SDH nucleic acid fragments were isolated from several other plant species including wheat and rice by identifying EST sequences homologous to the already known plant LKR/SDH sequences.

7. Fourth, there is a description of how to use these nucleic acid fragments to practice the invention. In the case of LKR/SDH, the availability of plant LKR/SDH genes made it possible to block expression of the LKR/SDH gene in transformed plants via antisense inhibition or cosuppression. It was stated in the Office Action on page 4 that antisense inhibition and cosuppression of a gene in a plant is unpredictable. This is true only in the sense that every transformant does not produce the desired phenotype. But one skilled in the art is well aware of this and designs the experiment in a way that many transformants are obtained and screened for the desired phenotype.

My own experience with cosuppression methodology in plants, as well as my knowledge of the work of my colleagues, and research work in the broader scientific community, indicates that this method is reliable and predictable. The use of cosuppression to block expression of several different genes in several different plants has been achieved [quite] successfully at DuPont.

Specifically in the case of LKR/SDH, cosuppression has been used to block expression with the first gene fragment and promoter combination tested, which hardly represents undue experimentation (see point 10 below).

8. It is stated on page 5 of the Office Action that "De Luca teaches that modifying metabolic pathways by transforming plants with genes that control steps of the pathway is highly unpredictable and often the desirable results are impossible to achieve." This may be true in cases where not enough is known about the metabolic pathway, but in the case of the lysine biosynthetic and catabolic pathways, it has been demonstrated how to increase production of lysine via modification of the biosynthetic pathway using lysine insensitive DHDPS and AK, and shown that accumulation of free lysine in seeds is also controlled by catabolism of lysine. We teach that blocking the first step in lysine catabolism will lead to increased accumulation of lysine and this is, in fact, what we have observed as described below.

9. The corn LKR/SDH cDNA sequence was used to identify transposon mutations in the endogenous corn LKR/SDH gene via PCR screening of a library of corn lines containing Robertson's Mutator transposon insertions. The precise location of Mutator insertions into the LKR/SDH gene was determined by sequencing of genomic DNA from individual mutants. An insertion mutation located in an exon in the LKR domain of the gene was chosen for further study. Southern blot analysis of corn genomic DNA indicated that corn contains only one LKR/SDH gene. Since an insertion mutation is expected to block function of the gene, it was anticipated that such a mutation would be recessive. One fourth of the progeny seed from a selfed corn ear with such a mutation segregating would be expected to be homozygous for the mutation. It was observed that approximately one fourth of such seed exhibited a higher level of free lysine than normal (5 to 15 fold higher) without the increase in the lysine catabolite saccharopine that is seen when free lysine is increased via expression of lysine insensitive DHDPS. It was concluded that knocking out LKR/SDH, by itself, was able to increase seed lysine content in corn seeds.

The LKR/SDH Mutator insertion line was crossed by a transgenic line that accumulates excess free lysine due to expression of lysine insensitive DHDPS and AK. In this cross two genetic loci that affect lysine accumulation, one of which is recessive (the LKR/SDH Mutator insertion) and one of which is semi-dominant (the lysine insensitive DHDPS and AK trangene locus), are segregating. Single seeds were analyzed for lysine and saccharopine content. The most striking observation from this experiment is that the highest lysine containing seeds have low levels of saccharopine (see figure). The low saccharopine level indicates that these seeds are homozygous for the LKR/SDH Mutator insertion, while the high lysine level indicates that they carry the lysine insensitive DHDPS and AK trangene locus. The level of lysine accumulation is considerably higher (2-3 fold) than the level provided by the

DHDPS and AK trangene locus alone. Thus, this experiment demonstrates that an increase in the accumulation of lysine, accompanied by a reduction in accumulation of lysine catabolites can be accomplished by combination of lysine overproduction brought about by expression of lysine insensitive DHDPS + AK and reduction of lysine catabolism by blocking expression of LKR/SDH, as we taught in the patent application. These results show that the concern stated in the Office Action on page 5 that "modifying metabolic pathways ... is highly unpredictable and often the desirable results are impossible to achieve" is unfounded in this particular case.

10. As indicated above, LKR/SDH expression has been blocked in corn via cosuppression. To accomplish this a chimeric gene designed for cosuppression of LKR was constructed by linking a 1268 bp LKR/SDH gene fragment, which included the LKR coding domain, to the corn endosperm 27 kD zein promoter and 10 kD zein 3' untranslated region. This chimeric gene was introduced into corn by particle-gun mediated transformation. Of 72 transformation events that were regenerated into plants and produced seed, 13 had seeds with a greater than four fold increase in free lysine. This is a typical frequency for cosuppression events. Since the transformed plants were out-crossed, the transgenic locus must be dominant or there would not have been any observable phenotype. This is expected from a cosuppression transgene, and is an advantage over knock-out mutations like the LKR/SDH Mutator insertion described above.

Some of the LKR cosuppression transformants have been carried forward for further testing. An event that has continued to show the increased free lysine phenotype for several generations and behaves genetically as a single locus transgene insertion has been selected for crossing to the transgenic line that accumulates excess free lysine due to expression of lysine insensitive DHDPS and AK. Results from that experiment are not yet available, but the expectation is that seeds carrying both transgene loci will have higher lysine levels than either parent, as was observed in the LKR Mutator insertion cross described above. In addition, co-transformation experiments in which the chimeric gene designed for cosuppression of LKR described above has been combined with a chimeric gene for expression of lysine insensitive DHDPS and introduced into corn by particle-gun mediated transformation are in progress. This is expected to yield transformants that produce seeds with the high lysine level observed in the LKR Mutator insertion cross by lysine insensitive DHDPS and AK, but with both chimeric genes at a single genetic locus, which is highly desirable for corn breeding.

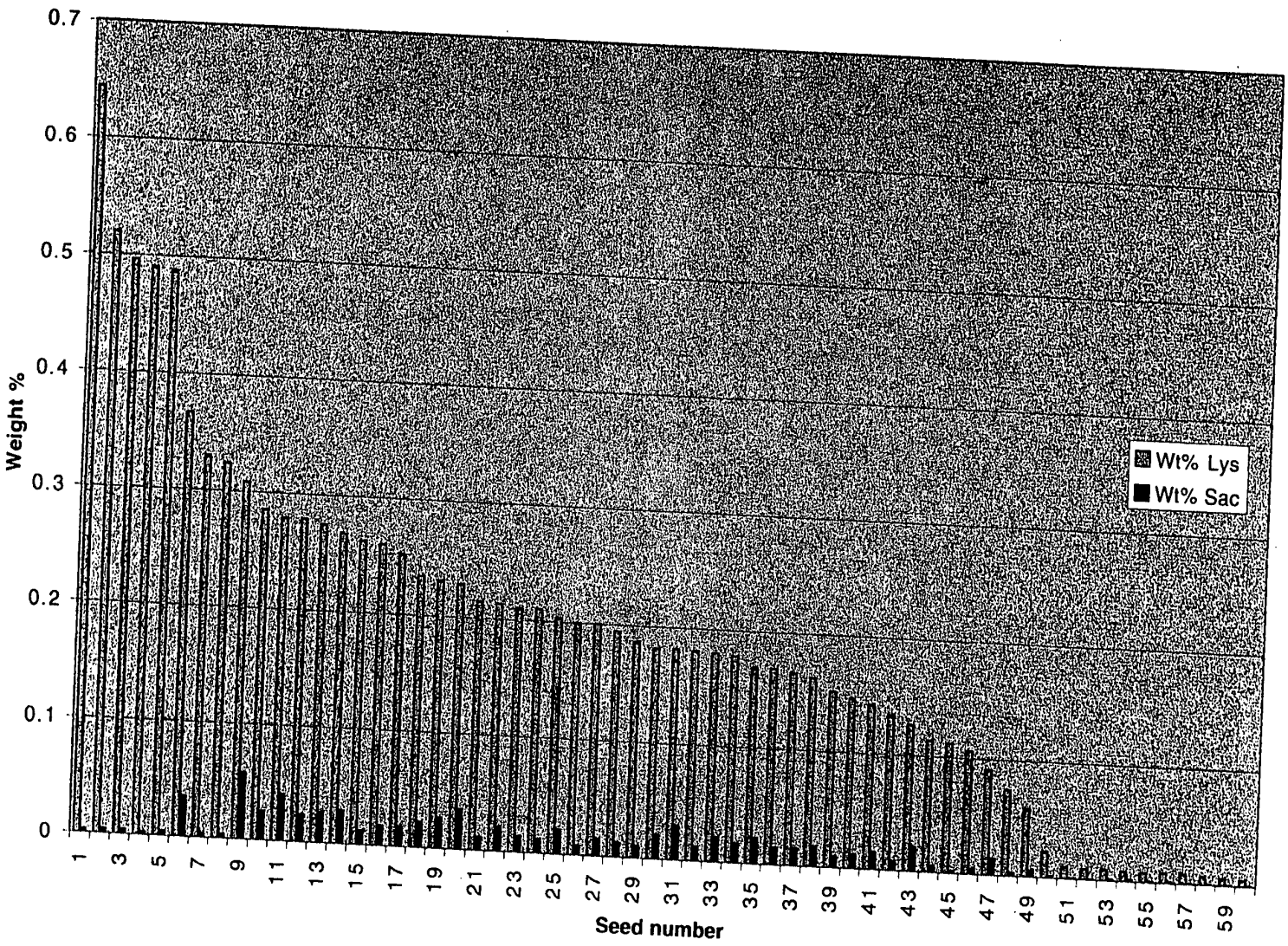
In summary, all of the elements of the claimed invention were provided in the patent application. The teachings in this case are in the public domain, due to the issuance of U. S. Patent 5,773,691 of which the instant application claims priority as a divisional application.. One skilled in the art can take these elements, as discussed above, and practice the invention without undue experimentation.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Saverio Carl Falco

8/24/00

Date


Lysine & Saccahropine in (DHDPS + AK) x (mu::LKR) seeds
Weight %
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Wt% Lys
Wt% Sac
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
33
35
37
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41
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Seed number